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Evidence for Avian and Human Host Cell Factors That Affect the Activity of Influenza Virus Polymerase▽†

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Typical avian influenza A viruses do not replicate efficiently in humans. The molecular basis of host range restriction and adaptation of avian influenza A viruses to a new host species is still not completely understood. Genetic determinants of host range adaptation have been found on the polymerase complex (PB1, PB2, and PA) as well as on the nucleoprotein (NP). These four viral proteins constitute the minimal set for transcription and replication of influenza viral RNA. It is widely documented that in human cells, avian-derived influenza A viral polymerase is poorly active, but despite extensive study, the reason for this blockade is not known. We monitored the activity of influenza A viral polymerases in heterokaryons formed between avian (DF1) and human (293T) cells. We have discovered that a positive factor present in avian cells enhances the activity of the avian influenza virus polymerase. We found no evidence for the existence of an inhibitory factor for avian virus polymerase in human cells, and we suggest, instead, that the restriction of avian influenza virus polymerases in human cells is the consequence of the absence or the low expression of a compatible positive cofactor. Finally, our results strongly suggest that the well-known adaptative mutation E627K on viral protein PB2 facilitates the ability of a human positive factor to enhance replication of influenza virus in human cells.

The natural hosts of influenza A virus are wild waterfowl, where typically infection occurs asymptomatically. These species provide a vast reservoir for maintenance and persistence of the virus in nature. In humans, avian influenza viruses usually do not replicate efficiently or cause disease (2). The recent H5N1 genotype Z-based viruses are a notable exception. Most transmissions of whole avian influenza viruses from birds to humans do not result in sustained circulation in humans (54), indicating that, in order to become endemic in the human population, avian influenza viruses must overcome host range restriction. Adaptation to humans is a rare but recurrent event that results in an influenza pandemic, followed by the establishment of a new lineage of human viruses. In 1918, 1957, and 1968, influenza pandemics arose when avian influenza viruses became adapted for the human host by mutation or recombination with currently circulating human viruses (54). In 2009, a new reassortant influenza A virus strain emerged from swine in Mexico that was able to infect and cause disease in humans, as well as to transmit infection between them. Following the rapid dissemination of the virus across the globe, the WHO announced the first influenza virus pandemic in 40 years (11). Although some genes of this pandemic H1N1 strain were originally derived from avian influenza viruses, including key components of the polymerase complex, it is likely they have undergone genetic adaptation in swine that allows the efficient replication of the pandemic virus in humans (31).

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The restriction of efficient infection of humans by avian influenza viruses might occur at various points in the virus infectious cycle. The mucus secreted by airway cells (37), the avian influenza receptor preference [sialic acid attached to galactose by an α(2,3) linkage] (29), and α(2,3) sialic acid distribution (24, 45) are innate barriers that can explain the restriction of avian viruses in the human respiratory tract. Although immunity to currently circulating human H1 and H3 viruses may explain the restriction of avian viruses of these subtypes, it does not explain why other subtypes (i.e., H4, H6, H9, and H10) showed only limited replication in human volunteers (2). Also, we along with others recently showed that avian influenza viruses are restricted by the low temperature of the human proximal airways (16, 43).

Even if the avian virus can enter the human cell, it is confronted with important changes in the cellular microenvironment that might influence every step of the replication cycle. These could be manifest as the absence in the human cell of a cellular factor upon which the virus depended or the presence in the human cell of an inhibiting factor that must be circumvented. Even subtle changes such as a lower or higher concentration of, or affinity for, a positive or a negative factor could impede the replication of the avian influenza virus in the human cell.

Influenza virus genome replication takes place in the host cell nucleus; it is essential that the viral ribonucleoprotein (vRNP) complexes gain efficient entry through the nuclear pore complex by interacting with transport proteins such as the importins (53). It has been suggested that the avian virus polymerase subunit PB2 and nucleoprotein (NP) may bind less efficiently to human importins than the proteins of viruses adapted to humans. Thus, slower accumulation of the vRNPs inside the nucleus may account for the lower replication of avian viruses in human cells (12, 42). Indeed, the point muta-

tion D701N in the PB2 polymerase subunit that can adapt an avian virus to human cells likely affects the interactions between PB2 and human importin α (50, 51). It may also be that the interaction with importin is important for polymerase function, independent of its role in nuclear transport (42). Once inside the nucleus, the viral RNA-dependent RNA polymerase must transcribe and replicate the eight viral genomic segments. Genetic and bioinformatics analysis has identified host-associated signatures on several of the viral genes that are components of the vRNP (5, 26, 33, 49).

Many studies have identified the viral protein PB2 as a strong determinant of influenza virus host range in tissue culture and mice (1, 10, 47; reviewed in reference 36). A particularly remarkable host-associated genetic signature is located at residue 627 of PB2. Human viruses generally have a lysine residue (rarely an arginine) at this position, whereas avian viruses have a glutamic acid. In mammalian cells, a reassortant virus that derived its PB2 from an avian influenza virus and remaining genes from a human influenza virus was restricted in replication (6). By passage through mammalian cells, this restricted virus acquired the ability to replicate by acquisition of the PB2 E627K mutation (48). The 1918 pandemic virus had a lysine residue at position 627 although the PB2 gene was otherwise avian virus-like, suggesting that selection of this adaptive mutation may have occurred early in the emergence of the Spanish flu pandemic (52). H5N1 viruses isolated from humans in Hong Kong in 1997 differed at this position, and there was a strong association of a lysine at position 627 with fatal outcome in mice (13, 15), whereby the presence of a lysine led to more aggressive viral replication in a variety of organs in the infected animals (46). Moreover, lysine 627 and asparagine 701 were both correlated with fatal disease in patients infected in Vietnam with H5N1 viruses in 2004 and 2005 (7). However, the molecular mechanism by which the nature of residue 627 on PB2 modulates replication of avian influenza viruses in human cells is not understood. The attenuated replication of avian influenza virus at a cooler temperature was somewhat relieved by the E627K mutation (16, 28). In addition, the formation of the RNP complexes from avian virus-derived polymerase was impaired in human cells but restored by the E627K mutation (25, 32, 41).

It was recently suggested that a dominant inhibitory activity present in human cells selectively restricts activity of a human influenza virus polymerase containing the "avian" signature E627 on viral protein PB2 (32). Using a similar approach, we investigated whether factors in human or avian cells might affect the human host range restriction of a typical avian virus-derived polymerase complex. We set up a replication assay in avian and human cell heterokaryons in which two different virus-like minireplicon RNA reporters (minigenomes) were transcribed by a viral polymerase complex reconstituted from expression plasmids. The reporter minigenomes were produced from species-specific polymerase I (Pol I) promoters active in the nuclei of the two different cell types. This assay allowed us to simultaneously monitor the avian virus polymerase activity in both types of nuclei in the context of a mixed avian and human cell environment. If a dominant negative factor were present in human cells, the reporter signal from both human and avian nuclei should be inhibited in hybrid cells. Conversely, if a positive cofactor were present in avian cells but absent in human cells, the human-specific reporter expression should increase. Finally, a situation in which the human reporter is not rescued and the avian reporter is expressed could be attributed either to a human restriction factor being strictly nuclear or to a required avian cofactor being exclusively nuclear. Surprisingly, our data indicate that there is no restriction factor for avian-derived polymerase present in human cells. Thus, the restriction in human cells is likely the consequence of the absence of an interaction or a low-affinity interaction with an essential cofactor. Finally, this work also gives an explanation for the natural selection of the adaptative mutation E627K in PB2 by implying the presence of a human-specific positive cell factor that enhances replication of polymerases only when the PB2 627K motif is present.

MATERIALS AND METHODS

Cell lines and tissue culture. 293T, DF1, and CEF cells were cultivated in Dulbecco's modified Eagle medium (DMEM) with pyruvate and L-glutamine (Invitrogen), supplemented with penicillin and streptomycin, and 10% (vol/vol) heat-inactivated fetal calf serum (Biosera). Cells were kept at 37°C and 5% CO_2.

Minireplicon assay (comparison of 50-92 and Victoria in 293T cells). 293T cells were transfected in 24-well plates with plasmids encoding the PB1, PB2, PA, and NP proteins derived from the 50-92 or Victoria virus (NP, 160 ng; PB1 and PB2, 80 ng; PA, 20 ng,) together with a negative-sense firefly luciferase-expressing plasmid (pHuman-PolI-Firefly; 80 ng) using Lipofectamine 2000 transfection reagent (Invitrogen). At various times posttransfection, cells were lysed with 200 μl of reporter lysis buffer (Promega), and firefly luciferase activity was measured using an Autolumat Plus LB 953 (Berthold).

Fusion assay between 293T and DF1 cells. 293T and DF1 cells were transfected in six-well plates with polyethylenimine (PEI) transfection reagent with 1 μg of enhanced green fluorescent protein (eGFP) expression plasmid (pCH-GFPW) or 0.2 μg each of measles virus fusion protein (F) and hemagglutinin (H) expression plasmids (Schwarz strain; kindly provided by M. Pizzato). At 3 h after transfection, cells were washed once with phosphate-buffered saline (PBS) and detached with 300 μl of 0.01% trypsin–0.04 g/liter EDTA. After a 5-min incubation at 37°C, trypsin was neutralized with 300 μl of DMEM–10% serum. Cells were collected, centrifuged for 5 min at 60 × *g* at room temperature, resuspended with DMEM–10% serum supplemented with antibiotics, counted, and mixed in a 12-well plate (ratio of 1:1). Cells were incubated at 37°C with 5% CO_2 for 24 h.

Plasmid constructs. Coding sequences for *Renilla* luciferase, eGFP, and DsRed were introduced into pCk-PolI-Firefly (kindly provided by L. Tiley) between EcoRI and HinDIII restriction sites. The reporter minigenome-encoding plasmids were then digested with XhoI and HinDIII to replace the avian polymerase I-type promoter (27) by the human polymerase I-type promoter (39). Thus, the reporter minigenome expressed in avian or human cells is strictly the same.

Minireplicon assay in fused 293T and DF1 cells. 293T cells were transfected in six-well plates using Lipofectamine 2000 transfection reagent (Invitrogen) with avian or human virus-derived polymerase and NP expression plasmids and a firefly luciferase minigenome reporter plasmid (NP, 0.8 μg; PB1 and PB2, 0.4 μg; PA, 0.1 μg; pHuman-PolI-Firefly, 0.4 μg). The 293T cells expressing a polymerase complex were named 293T-Pol. In parallel, DF1 and 293T cells were transfected with the two measles virus protein F and H expression plasmids (for DF1 cells, 0.4 μg each; for 293T cells, 0.2 μg each) and a *Renilla* luciferase minigenome reporter plasmid (0.8 μg of pCk-PolI-Renilla or 0.4 μg of pHuman-PolI-Renilla, respectively). Cells expressing the fusogenic proteins and a species-specific *Renilla* luciferase minigenome were named DF1-R and 293T-R. At 3 h after transfection, cells were washed once with PBS, detached as described above, and counted. A total of 2.5×10^5 293T-Pol cells were seeded in each well of a 24-well plate, and DF1-R or 293T-R cells were added (ratios of 293T-Pol to DF1-R or 293T-R were 60:1, 30:1, 15:1, and 7.5:1). Cells were incubated at 37°C with 5% CO_2 for 20 h and lysed with 200 μl of passive lysis buffer (Promega) to measure firefly and *Renilla* luciferase activities. When the fluorescent reporters were used, 293T and DF1 cells were transfected as described above, detached, and counted. 293T cells expressing the DsRed minigenome and 293T or DF1 cells expressing the 50-92 polymerase complex (PB1, PB2, and PA), 50-92 NP, an eGFP minigenome, and the measles virus F and H fusogenic proteins were mixed in a six-well plate (7.5×10^5 of each cell type per well). Cells were incubated at

37°C with 5% CO_2 for 20 h, washed with PBS, and fixed for 30 min at room temperature with 4% paraformaldehyde. Cells were stained with 4′,6′-diamidino-2-phenylindole (DAPI; 0.5 μg/ml) and analyzed for eGFP and DsRed expression using an Axiovert 40 confocal laser (CFL) microscope and an AxioCam MRc camera (Carl Zeiss).

RESULTS

Human cells do not support efficient replication of avian influenza virus polymerase complex. It is well accepted that avian influenza viruses do not replicate to their maximum capacity in most types of mammalian cells. In tissue culture this manifests as an inability to form plaques or to accumulate significant virus yield during multiple cycles of replication. Using a cell-based polymerase activity assay, we compared activities of polymerase complexes from an avian or a human influenza A virus in human (293T) cells. 50-92 is an H5N1 highly pathogenic avian virus (HPAI) isolated during an outbreak in domestic birds in Norfolk, England, in 1991 (A/Turkey/England/50-92/91) (55). Unlike modern genotype Z-like H5N1 viruses, there is no evidence that humans or other mammals were infected by the 50-92 virus or its derivatives during the HPAI outbreak. Victoria is a representative human H3N2 strain isolated in Australia in 1975 (A/Victoria/3/75).

In the minigenome assay, vRNP complexes are generated *in situ* in cells by expression of viral polymerase proteins (PB1, PB2 and PA) and the vRNA coating protein NP, together with a negative-sense virus-like RNA reporter (named minigenome) that has been transcribed by the cellular RNA polymerase I from a species-specific RNA polymerase I promoter. The reporter coding region of the minigenome is flanked by the viral conserved noncoding sequences that are the minimal viral promoters bound by the heterotrimeric polymerase. Viral replication and transcription of the negative-sense minigenome RNA take place in the nucleus and occur only in the presence of a reconstituted functional influenza virus polymerase (39).

Human 293T cells were cotransfected with plasmids expressing PB1, PB2, PA, and NP from 50-92 or Victoria virus, together with a firefly luciferase minigenome plasmid. Firefly luciferase activity was measured at different times after transfection (see Fig. S1 in the supplemental material). The avian influenza virus-derived 50-92 polymerase complexes did not replicate efficiently in the human cells, but polymerase activity was dramatically increased (by 2 logs) when the "human" signature (K627) was introduced into the PB2 protein. Conversely, the human virus-derived Victoria polymerase showed robust activity in human cells, but introduction of the "avian" signature E627 on PB2 resulted in a strong reduction in activity. These results are in agreement with previous studies showing reduced activities of polymerase complexes from avian virus in mammalian cells and the modulating role of PB2 amino acid 627. This effect was previously shown not to be due to a lower expression of PB2 627E protein (25, 28, 32).

Fusion of avian and human cells mediated by expression of measles virus glycoproteins. The molecular basis for the low efficiency of avian polymerases (or the PB2 K627E mutated human polymerases) in mammalian cells is not yet clear. To determine if this is due to the presence of a restriction activity or to the absence of an essential cofactor, we aimed to fuse avian cells with human cells in order to monitor activity of the avian virus polymerase complex in the presence of factors from both cell types.

To this end, we tested the ability of the measles virus fusion protein (F) and hemagglutinin (H) to induce fusion between DF1 and 293T cells. Plasmids encoding the F and H proteins were transfected into either human or avian cells while a plasmid encoding the eGFP was transfected into the other cell type to monitor cell fusion. As shown in Fig. S2C in the supplemental material, DF1 cells did not fuse together, which is likely explained by the absence of a receptor for measles virus at the surface of avian cells. As a consequence, there was no fusion between human and avian cells when F and H proteins were expressed from human cells (see Fig. S2D). However, when DF1 cells were expressing the two fusogenic proteins, an efficient fusion with human cells was observed (see Fig. S2E). Finally, 293T cells were very efficiently fused together when expressing F and H measles virus proteins (see Fig. S2F).

A positive cofactor for avian influenza virus polymerase complex in avian cells can complement avian polymerase function in human cells. The polymerase assay was carried out in hybrid cells formed between DF1 and 293T cells using the avian virus 50-92 polymerase complex. In this assay, both types of cells expressed a species-specific minigenome reporter.

Human cells were transfected with plasmids encoding PB1, PB2, PA, and NP as well as a negative-sense firefly luciferase RNA minigenome under the control of a human polymerase I (Pol I) promoter (pHuman-PolI-Firefly). Three hours after transfection, these cells (named 293T-Pol) were detached and mixed with increasing amounts of avian cells that were previously transfected with plasmids encoding measles virus F and H proteins and a plasmid expressing a minigenome with negative-sense *Renilla* luciferase RNA under the control of an avian Pol I promoter (pCk-PolI-Renilla) (DF1-R cells). Thus, firefly luciferase expression reflected activity of 50-92 polymerase complex in the nucleus of human cells, whereas the *Renilla* luciferase signal was the result of 50-92 polymerase function in the avian cell nucleus. A diagram of the experiment is presented in Fig. 1A. Results in Fig. 1B show that firefly luciferase expression driven by the 50-92 avian virus polymerase increased when the 293T-Pol cells were fused with increasing numbers of DF1-R cells (Fig. 1B, gray bars). This implies that supplying an avian factor enhanced the activity of the avian virus polymerase complex in the human nucleus. In parallel, the minigenome in the avian cell nucleus (*Renilla* luciferase reporter) was also expressed in a dose-dependent manner (see Fig. S3, gray bars, in the supplemental material), which proved that human and avian cells were successfully fused and that 293T-expressed 50-92 polymerase complex could enter the avian nucleus and function there efficiently even though it had been exposed to human cell factors. DF1 cells did not enhance expression of the minigenome reporter when mixed with human cells unless they were transfected with the two plasmids encoding the fusogenic F and H proteins (data not shown).

As a control, the same assay was performed in parallel but fusing 293T-Pol with 293T cells that were previously transfected with plasmids encoding measles virus F and H proteins and a plasmid expressing a *Renilla* luciferase minigenome under the control of a human Pol I promoter (pHuman-PolI-Renilla) (named 293T-R cells). Expression of the firefly luciferase minigenome by 50-92 polymerase in 293T-Pol was not altered after

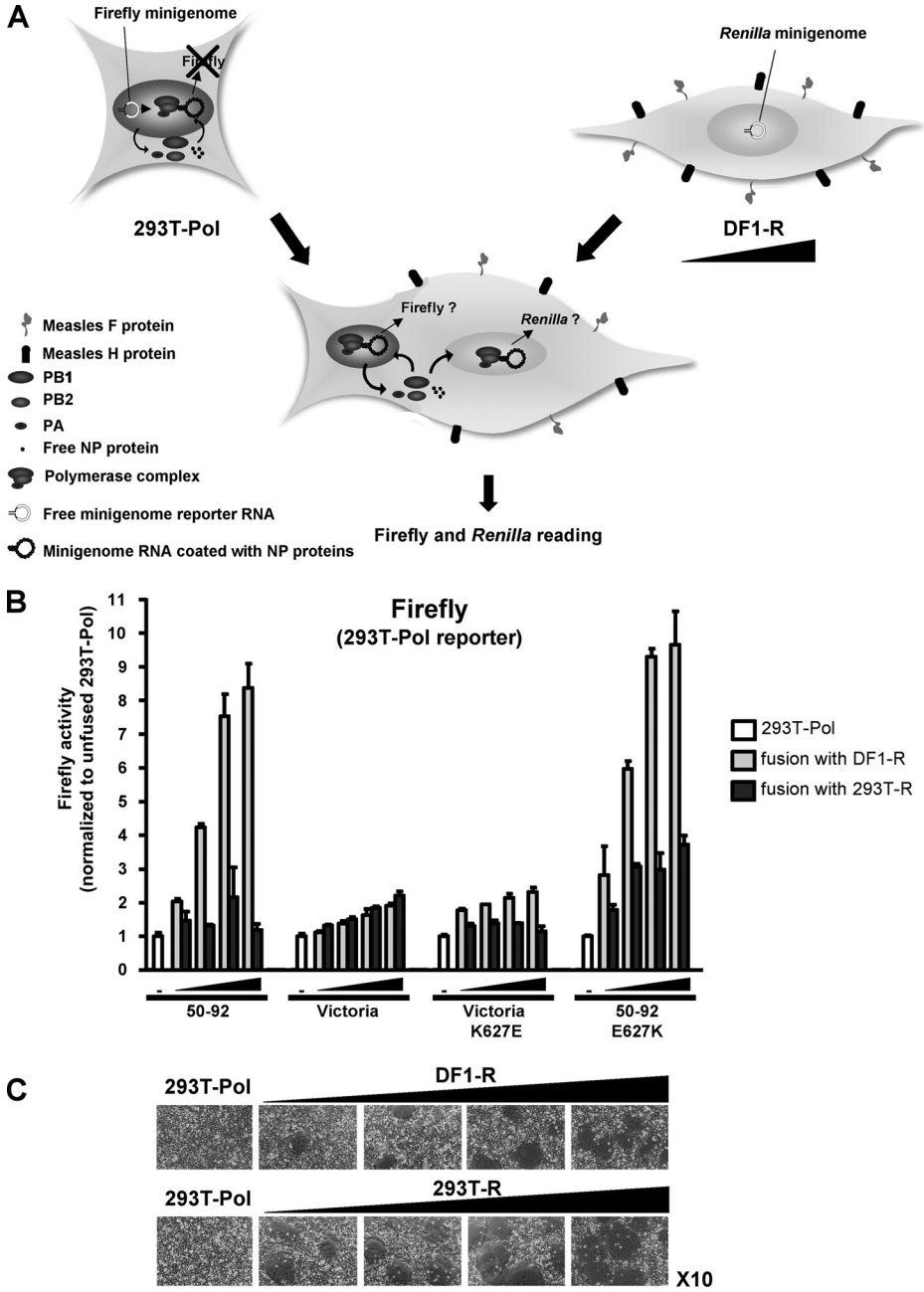

FIG. 1. Fusion of human cells with avian cells enhances replication/transcription activity of avian-origin influenza virus polymerase complex. (A) Diagram of the experiment. Influenza virus polymerase complex, derived from either 50-92 avian virus or Victoria human virus or with PB2 mutations at residue 627, and a firefly luciferase minigenome were expressed from 293T cells (293T-Pol) and fused with increasing amounts of DF1 cells (or 293T cells as control) expressing a *Renilla* luciferase minigenome and the two measles virus F and H fusogenic proteins (named DF1-R or 293T-R cells, respectively). Three hours after transfection, cells were detached and mixed. The number of 293T-Pol cells remained constant, whereas the amount of DF1-R (or 293T-R) increased. After incubation for 18 h, cells were lysed, and firefly and *Renilla* luciferase activities were measured, as represented in panel B and in Fig. S3 in the supplemental material, respectively. For each of the four different polymerase complexes tested, the firefly luciferase signals were normalized to the signal from unfused 293T-Pol (white bars). Firefly luciferase expression after fusion with DF1-R cells and after fusion with 293T-R cells is shown. Results were expressed as the mean ± standard deviation of duplicate samples from one experiment representative of three independent experiments. Ratios of 293T-Pol to DF1-R (or 293T-R) were 60:1; 30:1; 15:1; and 7.5:1. (C) Pictures taken just before cell lysis show an example of syncytia formation between 293T-Pol and DF1-R or 293T-R.

fusion between human cells (Fig. 1B, black bars). The *Renilla* luciferase signal in this experiment was very low and corresponded to the minimal activity of 50-92 polymerase complex in human cells (see Fig. S3, black bars, in the supplemental material). This proved that it was the addition of factors from the avian cell, rather than the fusion event itself, that enabled the 50-92 polymerase activity to drive expression of the *Renilla* luciferase reporter.

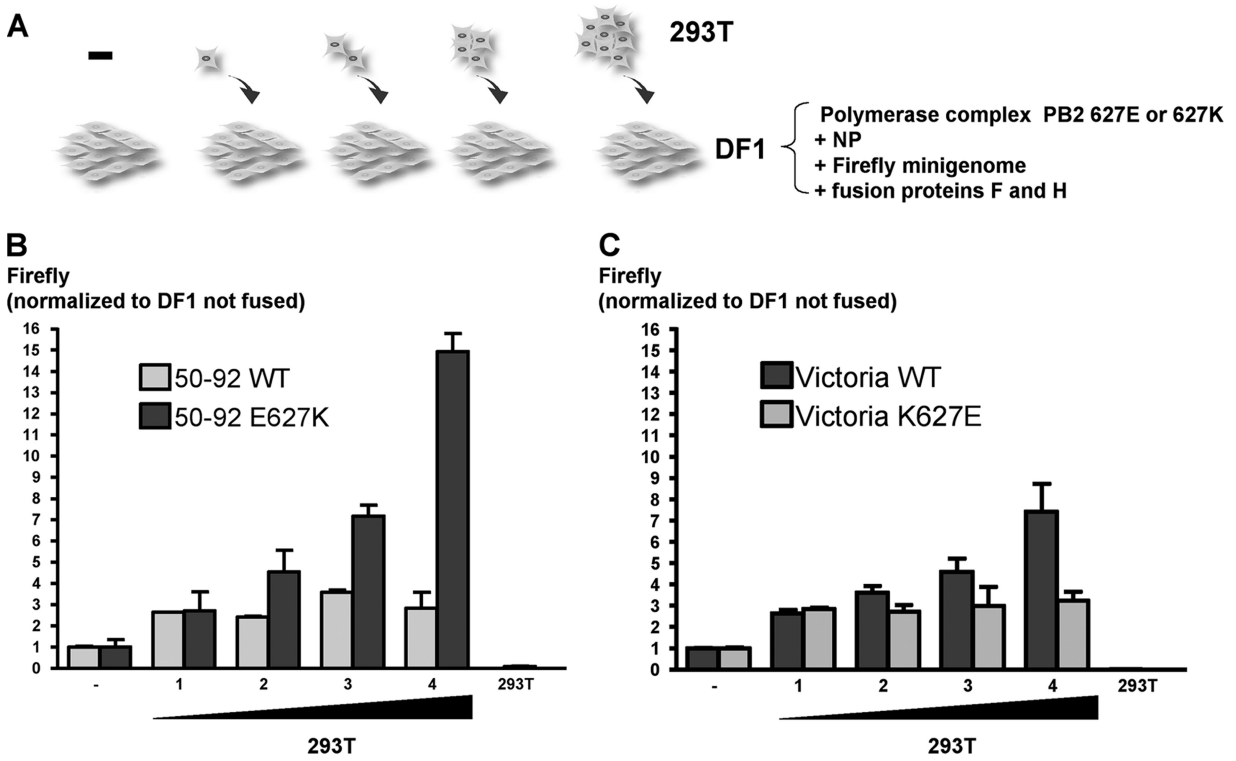

FIG. 2. The presence of a lysine in position 627 on PB2 is an adaptative mutation that facilitates polymerase enhancement by a human cofactor. (A) Diagram of the experiment. Polymerase complexes and NP were expressed from DF1 cells expressing a firefly minigenome and measles virus fusogenic F and H proteins. Three hours after transfection, cells were incubated with increasing amounts of 293T cells. After 18 h, cells were lysed, and firefly luciferase activity was measured. (B) DF1 cells expressing the avian 50-92 wild-type polymerase complex ([WT] PB2 627E) or harboring the human signature (PB2 E627K). (C) DF1 cells expressing the human Victoria virus wild-type (PB2 627K) or mutated (PB2 K627E) polymerase complex. For panels B and C, DF1 and 293T cells were mixed at a ratio of 60:1 (column 1), 30:1 (column 2), 15:1 (column 3), and 7.5:1 (column 4). Results were normalized to the control DF1 cells (−) not fused with 293T cells.

These results seem to be at odds with published data from Mehle and Doudna (32), who reported an inhibition of the A/WSN/33 virus polymerase bearing the avian PB2 motif E627 in the presence of human cell factors. We therefore repeated the experiment using a number of other polymerase configurations to drive expression of the minigenomes. We tested the activity of the human Victoria virus polymerase in heterokaryons, as well as that of Victoria polymerase with the PB2 point mutation K627E, and finally we included a 50-92 polymerase bearing the "humanizing" E627K PB2 mutation (Fig. 1B; see also Fig. S3 in the supplemental material). As seen for wild-type 50-92 polymerase, fusion with DF1 cells markedly enhanced the polymerase activity of the 50-92 627K polymerase (Fig. 1B, gray bars). On the other hand, the function of either of the Victoria-based polymerases was only marginally enhanced after fusion with avian cells. After 293T-293T fusion, polymerases with 627K in PB2 were somewhat enhanced, but polymerases with 627E were not (Fig. 1B, black bars). Moreover, the reporter present in DF1-R cells was expressed when any of the polymerases were generated in 293T-Pol cells (see Fig. S3, gray bars), which should not be the case if a dominant inhibitory factor that targeted the 627E PB2 polymerase complex were present in the human cells. As expected, fusion between 293T-Pol and 293T-R led to *Renilla* luciferase expression only with Victoria and 50-92 PB2 627K polymerases (see Fig. S3, black bars). An example of fusion between 293T-Pol and DF1-R or 293T-Pol and 293T-R cells is presented in Fig. 1C.

Finally, similar results were obtained using primary chicken embryo fibroblasts (CEFs) in place of DF1 cells (data not shown).

There is no dominant inhibitory activity that restricts avian influenza virus polymerase function in human cells, and a lysine in position 627 on PB2 leads to optimized replication in the presence of a human cofactor. In order to confirm the absence of any restrictive activity expressed from 293T cells, the reciprocal experiment in which chicken cells expressing the 50-92 avian virus polymerase complex and a firefly luciferase minigenome were fused with increasing amounts of human cells was carried out (Fig. 2). An inhibitory activity present in 293T cells should reduce firefly luciferase expression in a dose-dependent manner. However, the reporter expression was not inhibited by fusion with human cells. Rather, a moderate increase was observed (Fig. 2B, gray bars). This increase in signal may be due to the 293T cells allowing mixing of several different, partially transfected DF1 cells and increasing the chance that a complete polymerase complex might be present in the heterokaryons. The same experiment performed with the avian-like Victoria polymerase (PB2 K627E) gave similar results (Fig. 2C, gray bars) and confirmed the absence of a dominant restriction factor in 293T cells.

If the adaptive mutation E627K on PB2 is not a way to

escape from a human restriction factor, it may alternatively be selected because it creates a positive interaction with a human factor that increases replication activity of the virus polymerase complex. To test the effect of human factors on PB2 627K-containing polymerases, we tested in this assay 50-92 627K and Victoria virus polymerases. The increase in firefly luciferase expression driven by 50-92 polymerase complex harboring the E627K mutation on PB2 was much more dramatic and reached 15-fold when the ratio of 293T to DF1 was highest (Fig. 2B, column 4, black bar). When firefly luciferase expression was driven by Victoria polymerase complex, we again noted an increase of polymerase activity in fused cells when PB2 627K was part of the complex, in comparison with Victoria polymerase with a PB2 harboring the avian signature E627 (Fig. 2C).

Enhancement of influenza virus polymerase activity by avian host factors visualized at the level of individual transfected cells. To assess the level of polymerase activity within an individual heterokaryon rather than within the whole-cell population, we took the same approach as above but with two species-specific minigenomes that expressed fluorescent reporters: eGFP and DsRed. 293T cells transfected with a plasmid expressing a DsRed minigenome were mixed with either 293T or DF1 cells transfected with 50-92 PB1, PB2, PA, NP, measles virus F and H protein-expressing plasmids, and a plasmid expressing an eGFP minigenome under the control of species-specific Pol I promoter (pHuman-PolI-eGFP or pCk-PolI-eGFP, respectively). At 20 h after coculture, cells were analyzed for DsRed and eGFP expression. No red signal was observed from the control 293T cells expressing only the DsRed minigenome (Fig. 3A). 293T cells expressing 50-92 polymerase and the eGFP minigenome gave a low GFP signal (Fig. 3B), whereas bright green cells were observed from DF1 cells expressing 50-92 polymerase and eGFP minigenome (Fig. 3C). In fused 293T-DsRed/293T 50-92 Pol-eGFP cells, extensive syncytia formation was apparent by DAPI staining and light transmission images. However, the avian 50-92 polymerase complex drove only low expression of the eGFP minigenome reporter, and there was no red signal visible (Fig. 3D). However, when 50-92 polymerase complex was expressed by DF1 cells, a robust eGFP minigenome signal was evident in the heterokaryons (Fig. 3E). Note that since DF1 cells were not able to fuse together (see Fig. 2C in the supplemental material) and since 293T cells did not express the fusogenic proteins in this assay, syncytia were exclusively the result of fusion between DF1 and 293T cells. Moreover, after fusion of the human cells with avian cells, red fluorescence was observed, which was the result of viral transcription and expression of the DsRed minigenome reporter in the human nucleus. Finally, in a proportion of the fused cells, simultaneous expression of the two reporters was observed (Fig. 3E, white arrows). Thus, this result again confirms that there is no dominant restriction factor in human cells; otherwise eGFP expression would have been inhibited in those cells that were fused, and the human-avian cell heterokaryons would not have supported expression of the DsRed minigenome by the avian virus polymerase.

DISCUSSION

The host restriction of influenza virus is a multigenic trait determined by multiple interactions between viral components and host factors such as receptor expression, body temperature, and intracellular microenvironment that might vary between different species. Like all viruses, influenza A virus is an intracellular parasite that relies upon the cellular machinery for many aspects of its life cycle. Indeed, recent genome-wide screens have identified hundreds of cellular factors that affect virus replication or whose expression is modulated by the virus (3, 14, 20, 21, 23, 44). In particular, transcription and replication of influenza virus RNPs require interaction with many cellular factors, and a large number of human proteins that interact with the viral polymerase have already been identified biochemically in pulldown experiments (19, 30, 42) or by a yeast two-hybrid screen using human cDNA libraries (44). For example, PB1 and PA are imported into the nucleus as a dimer associated with RanBP5 (8). The MCM helicase has been shown to stabilize replication elongation complexes through scaffolding between nascent cRNA and viral RNA polymerase (22). The cellular splicing factor RAF-2p48/NPI-5/BAT1/UAP56 interacts with the N terminus of NP and facilitates formation of NP-RNA complex, stimulating viral RNA synthesis (34). Hsp90 was shown to interact with PB2 and enhance influenza viral RNA synthesis (35). Ebp1 (ErbB3-binding protein) was identified as a PB1 interactor and found to selectively interfere with *in vitro* RNA synthesis by influenza virus RNA polymerase (17). For these given examples, there has been no evidence that the interactions vary between species or could account for host range restrictions. Indeed, some of the interactions are known to occur even between human cell factors and the polymerase components of avian-adapted virus. For example, the cap-snatching mechanism that requires interaction of the viral polymerase complex with the cellular RNA polymerase II (9) is not impaired in human cells infected with an avian virus (41). The only RNP-host interaction so far associated with penetration of the host range barrier involves the nuclear import of NP and PB2 by interaction with importin α (38). At least one avian virus has overcome host range restriction by mutations in theses two viral proteins that increased their interactions with human importins and, consequently, the efficiency of their nuclear import (12).

Variation in the interactions between the PB2 polymerase subunit of human or avian-adapted influenza viruses and putative host factors offers the most plausible explanation for host range restriction. In 1977 Almond first showed that the PB2 gene segment was a key determinant for replication in mammalian cells. In 1993, Subbarao and coworkers showed that the amino acid 627 on polymerase subunit PB2 played a crucial role in influenza virus replication in mammalian cells (48). Since then, the involvement of residue 627 in overcoming mammalian host range restriction has been intensively studied, but the mechanism remains a puzzle. We obtained data showing that the host range restriction of avian RNPs in mammalian cells is not due to a restriction factor that specifically targets a glutamic acid in position 627 of PB2, the amino acid found in almost all avian influenza virus strains. We also found that a positive factor present in avian cells enhances replication/transcription of influenza virus RNP from avian origin. Thus, our conclusion is that an important cofactor required for the function of avian influenza virus polymerase is missing or underexpressed in human cells and that the PB2 E627K mutation can compensate for its absence by creating an adaptative in-

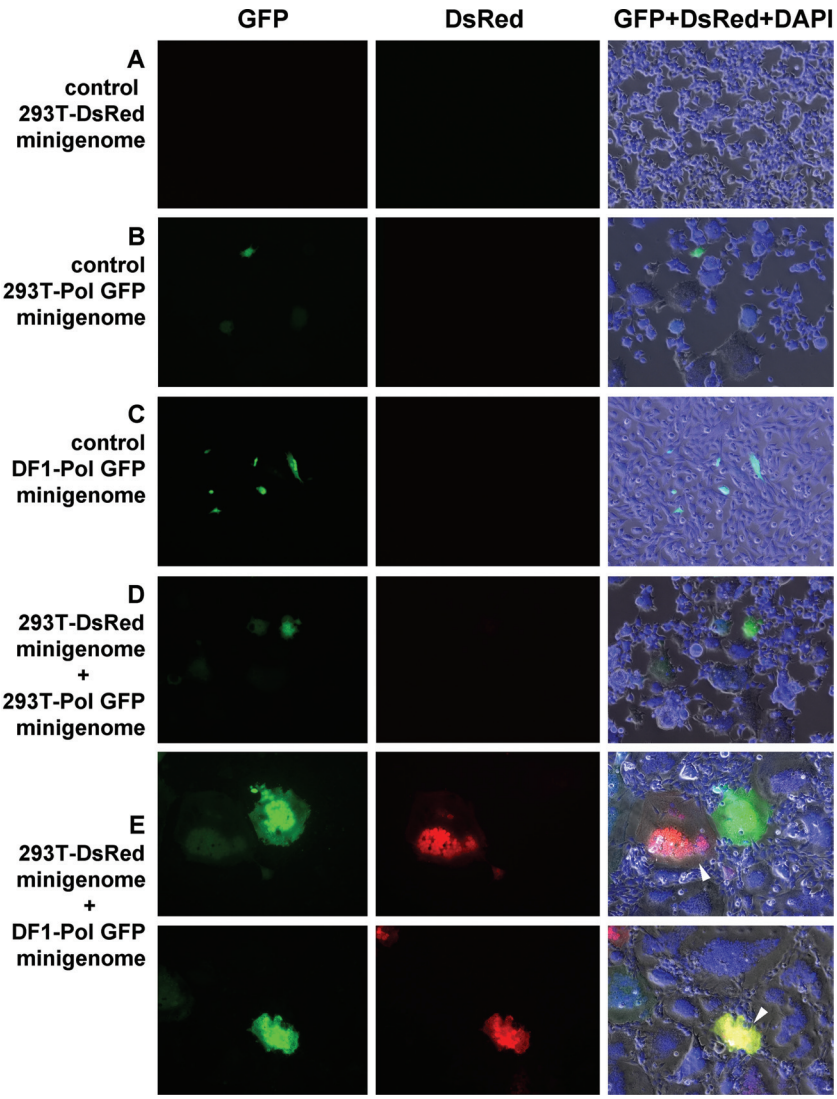

FIG. 3. Measurement of influenza virus polymerase activity in a fusion assay using two fluorescent reporters. 293T cells were transfected with a DsRed minigenome. At 3 h after transfection, cells were detached and mixed (ratio of 1:1) with 293T or DF1 cells expressing the 50-92 polymerase complex and NP, an eGFP minigenome, and the two measles virus F and H fusogenic proteins. Pictures were taken 24 h after coculture. (A) 293T cells expressing a DsRed minigenome. (B) 293T cells expressing the avian 50-92 polymerase and a GFP minigenome. (C) DF1 cells expressing the avian 50-92 polymerase and a GFP minigenome. (D) 293T cells expressing the DsRed minigenome fused with 293T cells expressing the 50-92 polymerase and the GFP minigenome. (E) 293T cells expressing the DsRed minigenome fused with DF1 cells expressing the polymerase and the GFP minigenome. Two different fields are shown. White arrows indicate syncytia in which the two minigenomes are expressed.

teraction with a cofactor present in human cells. Indeed, this hypothesis was supported by the observation that 293T cells supplied a cofactor that specifically increased activity of a PB2 627K-containing polymerase (Fig. 2).

Interestingly, using a similar approach, Mehle and Doudna came to a different conclusion: their recent publication (32) attests to the presence of a dominant restriction factor in human cells that inhibited the avian virus polymerase. The authors compared activities of the laboratory-adapted WSN strain in its wild-type form or mutated in PB2 K627E and concluded that the activity of the K627E PB2 polymerase did not approach that of wild-type polymerase in fused 293T-DF1 cells because of an inhibitor present in human cells. Our interpretation is different. We were able to show in a dose-response assay that fusion of human with avian cells did not inhibit an avian or an "avianized" polymerase. Moreover, an avian virus polymerase complex that was generated in human cells and therefore exposed to human factors still became active if it gained access to the avian nucleus. The lack of inhibition of polymerase function by human cell factors was particularly obvious when fluorescent reporters were used (Fig. 3). Finally, we were unable to saturate any restriction factor by transfecting excess plasmids encoding the 50-92 polymerase complex or 50-92 PB2 wild-type protein alone (data not shown). Thus, we concluded that there is no inhibitory factor that specifically targets the avian virus polymerase in human cells. Since the 627E polymerase was never as efficient even in avian cells as the 627K polymerase, it is not surprising that a

PB2 627E-containing polymerase did not reach the same absolute level of activity as a 627K-containing polymerase in fused 293T-DF1 cells. Interpretation of these data can be fraught with difficulty; it is hazardous to directly compare the widely opposed activities of polymerases harboring a lysine or a glutamic acid in position 627 in PB2 in a human cell. Instead, we suggest that comparison of polymerase activity between fused cells of the same or different origins as used in our studies may be a clearer way to investigate the presence or absence of a restriction factor.

The structural resolution of the carboxyl-terminal domain of the PB2 polymerase subunit has revealed that residue 627 sits on the surface of the molecule within a basic patch (51). It is apparent that the charge switch between the avian virus PB2 with glutamic acid and the human virus protein with lysine at this position would affect the presentation of the charged surface. If the purpose of this region is to repel a negative factor that is itself positively charged, then the lysine would indeed allow for that (31). However, an alternative explanation would be that the human host factor with which this domain interacts has a large negatively charged region with which the avian PB2 with its central glutamic acid can only weakly combine. If the role of this factor is to stabilize the NP-PB2 interaction within the RNP, then the weak interaction would account for the lack of coprecipitation of the two proteins from the RNP complex when they are derived from an avian virus and expressed in human cells (32, 41).

The dramatic increase of avian influenza virus polymerase activity in human cells when residue E627 is mutated to a K is likely attributed to the ability of the "humanized" PB2 protein to recruit a human positive factor. On the other hand, in avian cells virus polymerases containing PB2 627K are only two times more efficient than those with 627E (18, 32; also data not shown). This relative neutrality can be explained if the 627K positive factor has no homologue in avian cells or if residue 627 does not affect the interaction. It may be that different PB2 mutations are selected to optimize this virus-host interaction in different host species. Thus, while it is apparent that 627K is strongly selected for when an avian virus replicates in mice or humans (7, 15, 40), in equine or swine viruses 627K does not necessarily predominate (47). Thus, the 2009 swine-origin pandemic H1N1 virus, which efficiently replicates and transmits in humans and pigs (4), retains the avian signature E627 in PB2. Instead, it appears that two other PB2 residues, S590 and R591, are responsible for efficient activity of the pandemic virus polymerase in human cells (31). Based on the crystal structure of the PB2 domain comprised of residues 538 to 693, residues 590 and 591 lie very close to amino acid 627 (51), and we hypothesize that they may contact the same human cofactor as the PB2 containing lysine 627 to enhance the polymerase function. Whether other swine virus PB2 genes also have alternative mutations that mediate adaptation to the human cofactor remains to be seen.

The presence of highly pathogenic avian influenza viruses in Asia and Africa and the rapid spread of the new swine H1N1 across continents highlight the permanent threat posed by influenza virus. Thus, it is important to understand the interplay between the viral polymerase complex and host cell factors in order to understand which viruses are most likely to cross the species barrier. We hope that new insights into how the factors inside the human cell influence influenza A virus replication will lead to new strategies for inhibiting virus multiplication. The discrimination between the presence of a specific restriction factor and the absence of a cofactor for avian-derived viruses is an important step toward elucidating the mechanisms by which avian viruses might adapt to humans.

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